FRACTIONATION OF TRYPSIN BY PAPER ELECTROPHORESIS

By A. IACHAN, G. B. DOMONT, L. V. DISITZER and J. C. PERRONE

Laboratory of Protein Chemistry, Instituto Nacional de Tecnologia, Rio de Janeiro, Brazil

PREVIOUS work from this laboratory[1–3] has demonstrated the heterogeneity of crystalline trypsin by paper and starch column electrophoresis. This article presents an electrophoretic method of greater resolution. Using this technique it was possible to demonstrate in a sample of crystalline trypsin the occurrence of fractions showing different ratios of proteolytic to amidasic activity.

In the experiments recorded here a buffer of pyridine acetate of *p*H 4·9 (0·48 : 0·56 : 98·96 v/v of acetic acid : pyridine : water) was used for electrophoresis in a Durrum-type apparatus. To a strip of Whatman 3 filter paper of 12 × 50 cm, 0·12 ml. of an 8 per cent trypsin solution in 0·001 N hydrochloric acid were applied. An electrical tension of 450 V was used and the experiment was allowed to run for 5 h in the cold room. In these conditions at the end of the experiment the temperature of the buffer was 9° C. The strip, while still wet, was cut in 1-cm wide transversal segments and transferred individually to test-tubes containing 3 ml. of a 0·25 M pyridine formate buffer of *p*H 2·7. This extraction was allowed to continue for about 20 h in the cold room. The extract of each tube was then analysed for protein content, proteolytic activity against azo casein and amidasic activity towards α-benzoyl-L-arginine amide (BAA).

The protein content was determined by the Folin–Lowry[4] method as applied to the 'auto analyser' system[5]. Proteolytic activity against azo casein was measured by the method of Charney and Tomarelli[6]. The hydrolysis of BAA was followed colorimetrically in the auto analyser[7]. Chromatography of trypsin in carboxy methyl cellulose was carried out according to the method of Liener[8].

The trypsin samples used in this work were purchased from the Worthington Biochemical Corporation ('Cryst. Trypsin, lyophilized, lot *TL* 747–58'), Freehold, New Jersey, and from Novo Indústri ('Cryst. Trypsin Novo, batch 114–3'), Copenhagen, Denmark.

The results obtained by the fractionation of the Worthngton trypsin are shown in Fig. 1. The presence of three

Table 1

	Worthington trypsin				Novo trypsin			
	T*	F_1	F_2	F_3	T*	F_1	F_2	F_3
Proteolytic activity† (azo casein)	4·70	6·85	3·90	1·10	7·4	8·35	2·4	1·0
Amidasic activity‡ (α-benzoyl-L-arginine amide)	0·62	0·71	0·10	0·0	1·10	1·20	0·35	0·15
Ratio (proteolytic to amidasic)	7·8	9·5	39	—	6·7	6·9	6·8	6·7

* Unfractionated trypsin.

† The specific activity is given as the slope, multiplied by 10^3, of the straight line obtained by plotting the absorbance at 440 mμ against trypsin concentration in micrograms per millilitre.

‡ Same as above but plotting absorbance at 620 mμ.

distinct fractions is clearly shown, and all of them are active against azo casein. In the conditions of these experiments, however, the slower fraction (F_1) showed no activity towards BAA, while the faster fractions (F_2 and F_3) are active. The activity of each separated fraction is given in Table 1 and shows that the specific activity of F_3 for both substrates is higher than that of the unfractionated enzyme.

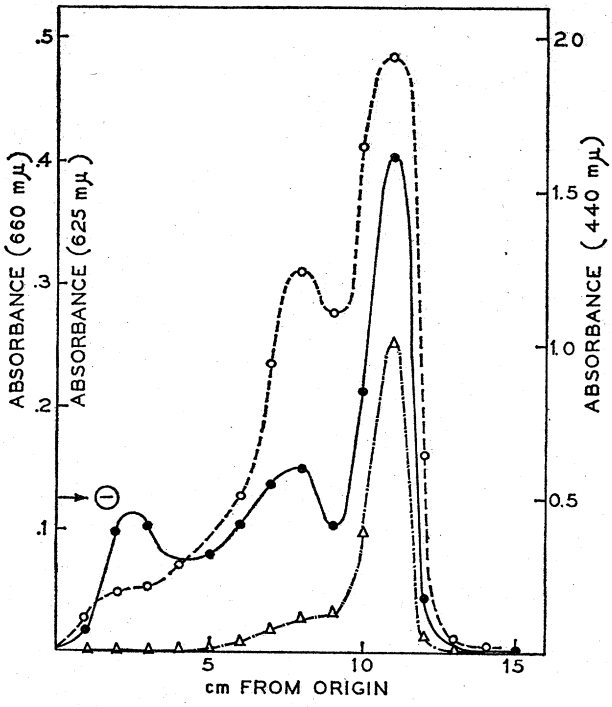

Fig. 1. Paper electrophoresis of a sample of Worthington trypsin. ●, Protein value (absorbance at 660 mμ); ○, proteolytic activity (absorbance at 440 mμ); △, amidasic activity (absorbance at 625 mμ)

The fact should be noted that the ratio obtained by dividing the proteolytic specific activity by the amidasic activity is different for each fraction. These results were observed only with the sample of Worthington trypsin.

These findings are consistent with those obtained by the electrophoretic fractionation of this same sample of trypsin in starch columns at *p*H 2·7 (ref. 3) which showed that the peaks representing the maximum of proteolytic activity and amidasic activity are not coincident.

The fractionation of Novo trypsin is presented in Fig. 2 and shows the appearance of only two fractions while the presence of a third slower fraction is only indicated. The specific activity of these three fractions is also given in Table 1 together with the calculated ratios of proteolytic activity to amidasic activity. These ratios for the sample of Novo trypsin examined are the same for all fractions obtained.

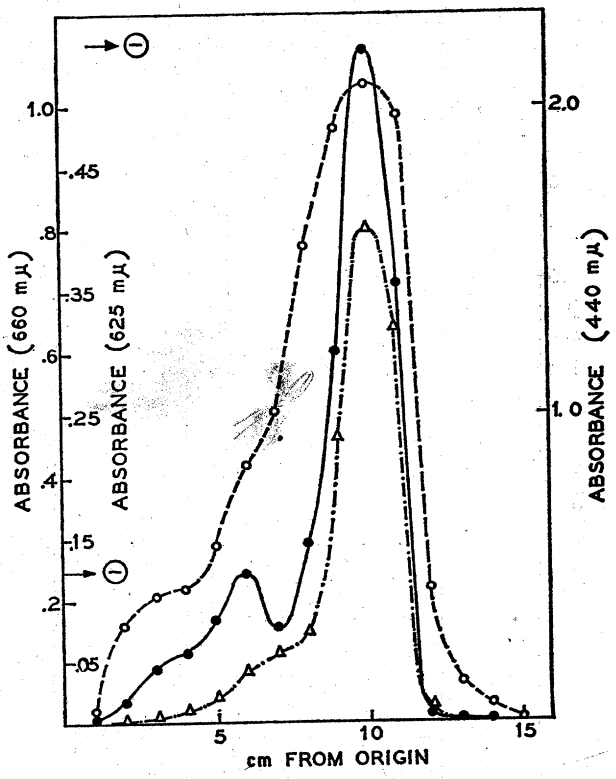

Fig. 2. Paper electrophoresis of a sample of Novo trypsin. ●, Protein value (absorbance at 660 mμ); ○, proteolytic activity (absorbance at 440 mμ); △, amidasic activity (absorbance at 625 mμ)

Electrophoresis of both samples of trypsin were also carried out in the presence of stabilizing concentrations of calcium. In this case calcium chloride was added to the H 2·7 buffer to obtain a 0·05 M concentration of Ca^{++}. The trypsin samples were dissolved in this calcium-containing buffer and incubated for 2 h at 37° C before electrophoresis. The fractionation diagrams obtained by electrophoresis of the calcium-treated samples of trypsin were in all respects similar to those recorded in Figs. 1 and 2.

In order to test a possible degradation of trypsin during the electrophoretic run the main fraction (F_3) was extracted, freeze dried and again submitted to the same electrophoretic fractionation process. The results of this experiment are recorded in Fig. 3 and show the presence of only one fraction which was found to be active against both substrates.

A search for degradation products was carried out by treating with ninhydrin the paper strip after the electrophoretic run. This test was also negative and showed that

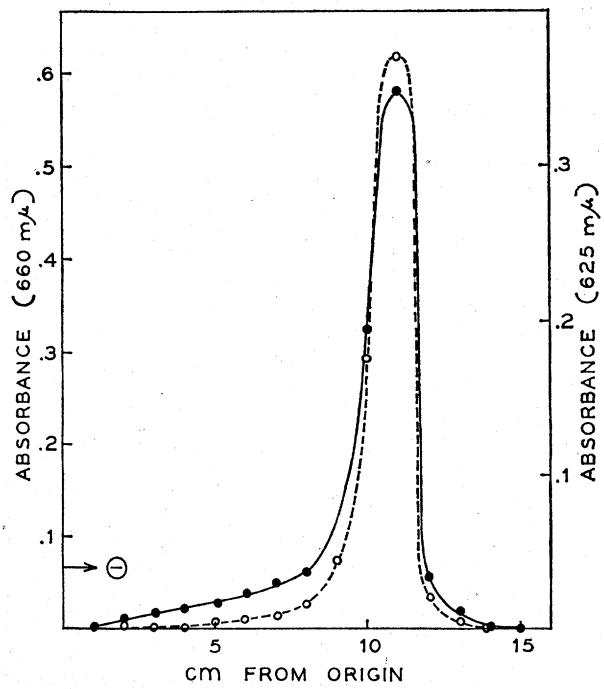

Fig. 3. Re-electrophoresis of the main fraction from the sample of Worthington trypsin. ●, Protein value (absorbance at 660 mμ); ○, amidasic activity (absorbance at 625 mμ)

no autolysis products reacting with ninhydrin were formed during electrophoresis.

These experiments tend to demonstrate that no degradation or formation of artefacts takes place during electrophoresis in the conditions employed in this investigation.

The appearance in the Worthington trypsin of fractions giving different ratios of proteolytic to amidasic activity could be explained by gross contamination of this sample with some other proteolytic enzyme such as chymotrypsin. However, the possibility of contamination with chymotrypsin was ruled out by paper electrophoresis at *p*H 2·7 (ref. 1). At this *p*H, chymotrypsin remains at the application point while trypsin migrates towards the anode, and when the Worthington trypsin was analysed by this method no proteolytic activity was found at the origin.

In order to correlate the results obtained with the present method of fractionation with those of Liener[8] using chromatography in carboxymethyl cellulose (CMC) the main fraction obtained from the Novo trypsin by this method was investigated by paper electrophoresis at

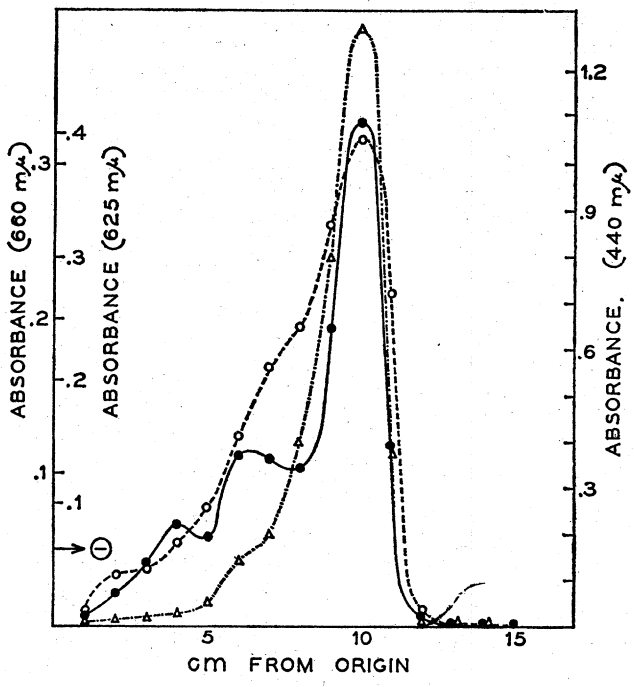

Fig. 4. Paper electrophoresis of the main trypsin fraction obtained by chromatography in carboxymethylcellulose. ●, Protein value (absorbance at 660 mμ); ○, proteolytic activity (absorbance at 440 mμ); △, amidasic activity (absorbance at 625 mμ)

*p*H 4·9. Prior to electrophoresis the main chromatographic fraction was dialysed in the cold against *p*H 2·7 pyridine formate buffer and freeze dried. The data obtained from this fractionation are recorded in Fig. 4 and show that the main fraction resulting from the chromatography of trypsin in CMC is heterogeneous when analysed by paper electrophoresis at *p*H 4·9. The fractionation diagram presented in Fig. 4 is similar to those obtained in the same conditions with the unfractionated enzyme.

The presence in crystalline trypsin of fractions each one showing a different ratio of proteolytic activity to amidasic activity was unexpected. One possible explanation for the observation would be the occurrence of limited autolysis of the enzyme during isolation. Changes in the specificity of trypsin following autolysis were observed by Bresler *et al.*[10]. Hakim *et al.*[11] have also obtained experimental evidence indicating that changes can occur in the specificity of trypsin following limited autolysis of this enzyme. The work of Viswanatha and Liener[12] is also pertinent to this question in showing that the specificity of the active fragment resulting from the peptic activation of acetyl trypsinogen was different from that of trypsin.

We have suggested in a previous article[3], based on the observations of Liener[8] and Gladner and Folk[9], that limited autolysis of trypsin resulting in the cleavage of lysine bonds near the trypsin C-terminal residue could account for the occurrence of the observed heterogeneity.

This work was supported in part by grants from the Foreign Research and Technical Programs Division, Agricultural Research Service of the U.S. Department of Agriculture (*FG–Br*–100) and the Conselho Nacional de Pesquisas.

[1] Perrone, J. C., Disitzer, L. V., and Domont, G., *Nature*, **183**, 605 (1959).
[2] Perrone, J. C., Disitzer, L. V., and Iachan, A., *Nature*, **184**, 1225 (1959).
[3] Disitzer, L. V., Domont, G. B., Iachan, A., and Perrone, J. C. (in the press).
[4] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, A. J., *J. Biol. Chem.*, **193**, 265 (1951).
[5] *Technicon Auto Analyzer Methodology, Protein Ib.*
[6] Charney, J., and Tomarelli, R. M., *J. Biol. Chem.*, **171**, 501 (1947).
[7] Perrone, J. C., Disitzer, L. V., Iachan, A., and Domont, G. B. (in the press).
[8] Liener, E., *Arch. Biochem. Biophys.*, **88**, 216 (1960).
[9] Gladner, J. A., and Folk, J. E., *J. Biol. Chem.*, **231**, 393 (1958).
[10] Bresler, S., Champagne, M., and Frenkel, S., *J. Chim. Physique*, **59**, 180 (1962).
[11] Hakim, A. A., Peters, R. L., Samsa, E. G., and Van Melle, P. J., *Enzymologia*, **25**, 134 (1962).
[12] Viswanatha, T., and Liener, I. E., *Biochim. Biophys. Acta*, **37**, 389 (1960).